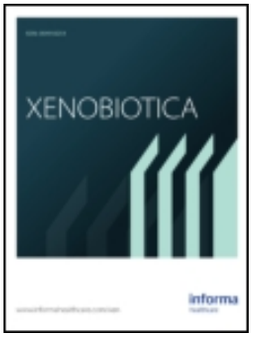

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Pharmacokinetics and metabolism of sildenafil in mouse, rat, rabbit, dog and man

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Pharmacokinetics and metabolism of sildenafil in mouse, rat, rabbit, dog and man

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1. Pharmacokinetics were studied in mouse, rat, rabbit, dog and man after single intravenous and/or oral doses of sildenafil or [^{14}C]-sildenafil (Viagra™).

2. In man, absorption from the gastrointestinal tract was essentially complete. With the exception of male rat, T_{max} occurred at ~ 1 h or less. Bioavailability was attenuated by pre-systemic hepatic metabolism in all species.

3. The volume of distribution was similar in rodents and humans (1–2 l/kg) but was greater in dog (5.2 l/kg), due to lower plasma protein binding (84 versus 94–96% respectively).

4. High clearance was the principal determinant of short elimination half-lives in rodents (0.4–1.3 h), whereas moderate clearance in dog and man resulted in longer half-lives (6.1 and 3.7 h respectively). Clearances were in agreement with *in vitro* metabolism rates by liver microsomes from the various species.

5. After single oral or intravenous doses of [^{14}C]-sildenafil, the majority of radioactivity was excreted in the faeces of all species. No unchanged drug was detected in the excreta of man.

6. Five principal pathways of metabolism in all species were piperazine *N*-demethylation, pyrazole *N*-demethylation, loss of a two-carbon fragment from the piperazine ring (*N*,*N*′-deethylation), oxidation of the piperazine ring and aliphatic hydroxylation. Additional metabolites arose through combinations of these pathways.

7. Sildenafil was the major component detected in human plasma. Following oral doses, AUC_∞ for the piperazine *N*-desmethyl and piperazine *N*,*N*′-desethyl metabolites were 55 and 27% that of parent compound respectively.

Introduction

Sildenafil (Viagra™, 1-[4-ethoxy-3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo[4,3-*d*]pyrimidin-5-yl) phenylsulphonyl]-4-methyl piperazine; figure 1) is a novel inhibitor of the human cGMP-specific phosphodiesterase type 5 enzyme (PDE5) found in human corpus cavernosum, discovered through a rational drug design programme (Terrett *et al*. 1996). The compound is relatively lipophilic (log $D_{7.4} = 2.7$) with a weakly basic centre in the piperazine tertiary amine ($pK_a = 6.5$). Sildenafil is a potent inhibitor of PDE5 with an IC_{50} of 4 nM and shows selectivity in potency over other PDE isozymes (Ballard *et al*. 1996). It has been shown to be effective as an oral (p.o.) treatment for male erectile dysfunction (Boolell *et al*. 1996).

To date, information on the disposition of sildenafil is limited to preliminary animal pharmacokinetic data (Rance *et al*. 1996) and p.o. pharmacokinetic data in human volunteers (Muirhead *et al*. 1996). In these studies, the piperazine *N*-

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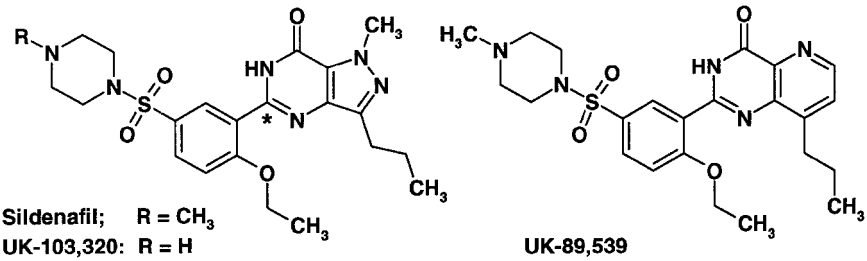

Figure 1. Structure of sildenafil, its metabolite, UK-103,320 (*position of [^{14}C] label) and internal standard, UK-89,539.

desmethyl metabolite (UK-103,320; figure 1) was identified as a major circulating metabolite in mouse, rat, dog and man. This paper reports the pharmacokinetics and metabolism of sildenafil in animals and man and attempts to rationalize the differences and similarities observed between species.

In the work described, bioavailability and pharmacokinetic studies have been conducted in animals using a specific hplc method to analyse sildenafil and UK-103,320 in biological fluids. Bioanalysis in man was conducted using automated sequential trace enrichment of dialysates (ASTED) and hplc (Cooper *et al*. 1997). In addition, radiolabelled sildenafil has been administered to laboratory animals and man to investigate its metabolic fate.

Materials and methods

Chemicals

Sildenafil citrate (UK-92,480), authentic samples of metabolites UK-103,320, UK-150,564, UK-95,340, UK-331,849 and UK-332,012 (figure 4) and internal standard, UK-89,539 (figure 1) were synthesized at Pfizer Central Research (Sandwich, UK). [Pyrimidine ring-2-^{14}C]-sildenafil was prepared by Amersham Intl (Amersham, UK) with a radiochemical purity > 97% (by radiochemical hplc) and a specific activity of 24 μCi/mg. The chemical purity of all compounds was 95% or greater.

Administration to animals

Sildenafil citrate or [^{14}C]-sildenafil citrate was dissolved in 0.1 N hydrochloric acid for intravenous (i.v.) and p.o. administration to mouse, rat, rabbit and dog. Five male beagle dogs (Pfizer colony, 12–17 kg) received sildenafil i.v. by infusion (5-min duration) into a saphenous vein at 1.0 mg/kg. The same animals received p.o. solution doses administered via gavage tube at 1.0 mg/kg. Doses were administered at least 7 days apart. Blood samples (5 ml) were collected from an indwelling saphenous vein cannula (opposite leg to that used for infusion) for the first 8 h and subsequently by venepuncture of the cephalic vein. One male and one female dog received [^{14}C]-sildenafil orally at 20 mg/kg containing ~ 200 μCi. Animals were housed in individual stainless steel metabolism cages (Modular Systems and Development Company Ltd) designed for separate collection of urine and faeces.

Ten female New Zealand White rabbits (2.2–2.8 kg, Froxfield Farm Ltd, Petersfield, UK) received single p.o. doses of [^{14}C]-sildenafil by gavage (50 mg/kg and ~ 40 μCi in 12 ml). Blood samples (6 ml) were collected from two animals per time point from the central ear artery, there being a total of four samples per animal up to 24 h. Three animals (not used for blood sampling) were housed individually in stainless steel metabolism cages adapted to enable the separate collection of urine and faeces.

Six male and six female Sprague-Dawley rats (~ 250 g, Charles River, Manston, UK) were surgically prepared with a jugular vein catheter at least 2 days prior to dose administration. Three male and three female animals received single i.v. doses into the caudal vein (4 mg/kg in 0.25 ml). Three male and three female animals received single p.o. doses by gavage tube (45 mg/kg in 2.5 ml). Blood samples (200 μl) were collected from the jugular vein catheter and the volume replaced with blood from a donor rat up to 12 h post-dosing. A final blood sample (5 ml) was collected at 24 h from the vena cava under halothane anaesthesia. In addition, non-cannulated male and female Sprague-Dawley rats (~ 250 g) received p.o. sildenafil by gavage tube (1 mg/kg in 1 ml). Blood samples (5 ml) were collected from the vena cava of two animals at each time point. Doses of [^{14}C]-sildenafil were administered by p.o. gavage (45 mg/kg and ~ 25μCi in 2.5 ml) to three male and three female Sprague Dawley rats, which were then housed in individual glass metabolism cages (Jencons Scientific Ltd) designed for collection of carbon dioxide, urine and faeces.

Male mice (CD1 strain, ~25 g, supplied by Charles River, Manston, Kent) were dosed with sildenafil by i.v. injection into the caudal vein (1 mg/kg in 0.1 ml) or by p.o. gavage (10 mg/kg in 0.5 ml). Blood samples (0.8 ml) were collected from the vena cava of five mice at each time point and pooled. Doses of [^{14}C]-sildenafil were administered by p.o. gavage (10 mg/kg and ~10μCi in 0.5 ml) to three male and three female mice which were then housed individually in glass metabolism cages (Jencons Scientific Ltd) designed for the separate collection of urine and faeces.

Urine and faeces samples were collected daily for 5 days from mice, rats and rabbits and for 6 days from dogs following dosing of radiolabelled compound. Urine was stored frozen at ~ −20°C before analysis. Faecal samples were homogenized with 2 vols water for mouse, rat and dog and 4 vols water for rabbit and the homogenate stored frozen at ~ −20°C before analysis. Respired air from rats receiving radiolabelled compound was passed through two traps containing 2 M sodium hydroxide. Aliquots of the trap fluid were retained for analysis of radioactivity. All blood samples were collected into lithium heparin tubes, mixed and centrifuged. After centrifugation, plasma samples were transferred to glass tubes and stored at ~ −20°C prior to analysis.

Administration to man

[^{14}C]-sildenafil (~50 μCi) was administered in solution orally (50 mg) or intravenously (25 mg) to six healthy male volunteers. The clinical study was conducted in compliance with the revised Declaration of Helsinki (1989) at the Besselaar Clinical Research Unit, Leeds, UK. The trial was reviewed and approved by the local ethics committee. The six volunteers provided written informed consent, were aged between 45 and 60 years and weighed between 60 and 90 kg. The subjects showed no evidence of any clinically significant disease, or clinically significant abnormality following review of laboratory data and full physical examination. Drug was administered i.v. as a constant rate infusion (1 ml/min) for 25 min. Blood samples (7 ml) were withdrawn from the forearm and transferred to lithium heparin tubes, mixed and centrifuged. Additional volumes of 50 ml blood were collected at 1 and 8 h post-dose. Following centrifugation, plasma samples were transferred to glass tubes and stored at −20°C until analysis. Urine and faeces were collected into polythene containers and stored at ~ −20°C until analysed.

Analysis of sildenafil in plasma samples from animals and man

The analysis of plasma samples for sildenafil and UK-103, 320, from rat, rabbit and man employed the automated sequential trace enrichment of dialysates (ASTED) and high-performance liquid chromatography (hplc) procedure of Cooper *et al.* (1997). UK-150,564 was also analysed in human plasma samples by a modification of this method. The analytical procedure for rat and rabbit plasma was validated by the analysis of quality control samples, prepared by the addition of sildenafil and UK-103,320 and stored frozen prior to analysis. The imprecision was < 15% at all concentrations (3–200 ng/ml) and the limit of quantitation was 1 ng/ml. The method used for the analysis of plasma samples from mice and dogs involved the addition of internal standard (400 ng UK-89,539) and mixing with 1 ml 0.05 M TRIS buffer, pH 9.0. The samples were applied to activated solid phase extraction cartridges (CH Bond Elut, Analytichem International) and the resin washed with the above TRIS buffer and 20% acetonitrile in water. Analytes were eluted with 1 ml methanol, evaporated to dryness under a nitrogen stream and resuspended in 100 μl methanol/water (60:40 v/v). Samples (80 μl) were injected onto the hplc column (Spherisorb 5 μ ODS 2 25× 0.5 cm, Hichrom, Reading, UK) with a mobile phase consisting of methanol/0.1 M TEMED phosphate, pH 5.0 (55:45 v/v) at 1 ml/min. Detection was by UV absorbance at 290 nm. The limit of detection of the assay was 5 ng/ml for sildenafil and UK-103,320 and analysis of samples of concentration unknown to the operator gave results within 10% of the correct value. Dog urine was also analysed by this method. The solid phase extraction method was used prior to the development of the ASTED method mentioned previously.

Pharmacokinetic analysis of data

Terminal elimination rate constant (k_{el}) was determined by linear regression of the log plasma concentrations. Area under the plasma concentration versus time curve (AUC) was calculated to the last time point from the linear trapezoidal method. AUC_{∞} was calculated from extrapolation to infinity with k_{el}. Clearance (Cl) was calculated by the relationship dose/AUC_{∞} and the volume of distribution was calculated by the relationship Cl/k_{el}.

Protein binding

Samples of mouse, rat, rabbit, dog and human plasma (1 ml) containing [^{14}C]-sildenafil (range 0.01–10 μg/ml) were dialysed (Spectrapor 1 dialysis membrane 6000–8000 molecular weight cut-off; Spectrum Medical Industries) against isotonic Krebs-Ringer bicarbonate buffer, pH 7.4 (1 ml) for 3 h at 37°C in a rotating dialyser (Dianorm, MSE). Following dialysis, concentrations of drug in plasma and buffer were measured by liquid scintillation counting. The plasma protein binding of the metabolite, UK-103,320, was determined at a single concentration of 1 μg/ml in plasma from each species. Dialysis

conditions were the same as those used for sildenafil and concentrations of UK-103,320 were determined by solid-phase extraction and hplc analysis (as previously described). For each species duplicate determinations were performed for sildenafil and UK-103,320.

Analysis of radioactivity

Radioactivity in samples (0.5 ml) of plasma and urine was measured by liquid scintillation counting in Lumagel (Rhône-Poulenc) or scintillation system MI31 (Packard Instrument Ltd). Radioactivity in weighed homogenized faeces samples (~ 0.2–0.4 g) was determined by liquid scintillation counting of evolved CO_2 after combustion of the samples in a sample oxidizer (Model 306/307, Packard Instruments Ltd).

Analysis of plasma for metabolites

The identity of human and animal plasma metabolites of sildenafil was investigated by analysis of plasma extracts by hplc. Plasma samples were extracted using C18 Bond Elut cartridges (Analytichem International) which were washed with 0.1 M HEPES buffer (pH 7) and eluted with methanol. Samples were evaporated to dryness under a nitrogen stream and reconstituted in 30% methanol:70% 0.05 M ammonium acetate. Each sample was injected onto a gradient hplc system comprising a 12.5×0.46 cm Spherisorb 5 μ ODS2 column (Hichrom) eluted by a gradient of 35–80% methanol in 0.05 M aqueous ammonium acetate over 30 min at 1 ml/min. The eluate was monitored for UV absorbance (230 nm) and by an in-line radioactivity monitor (β-Ram, Lablogic Ltd) or by fraction collection (0.25 min), mixing with 2 ml Starscint scintillation fluid followed by liquid scintillation counting. For identification of human metabolites, plasma samples (3 ml) from each volunteer were pooled at both the 1 and 8 h post-dose time points. Metabolites were extracted as described above and components separated on the gradient hplc system. Radioactive components were manually collected and analysed by mass spectrometry.

Analysis of urine for metabolites

For each species, gender and route of administration, pooled 0–24 h urine samples were prepared. These were extracted and analysed by a gradient hplc system comprising a 15×0.46 cm Spherisorb 5 μ ODS2 column (Hichrom) eluted by a gradient of 35–70% methanol in 0.05 M aqueous ammonium acetate over 60 min at 1 ml/min. Metabolites were isolated from a 10 ml pooled human urine sample by the same method described for plasma. These metabolites were further purified by isocratic hplc prior to mass spectral analysis.

Analysis of faeces for metabolites

Homogenized faeces samples were pooled proportionally from timed collections for each species, gender and route of administration for all samples containing > 5% of the administered dose. Faecal homogenates were extracted using 20% methanol:80% ethyl acetate. The extract was evaporated to dryness and resuspended in methanol, washed with cyclohexane and evaporated to a small volume, which was mixed with 30% methanol:70% water. This was extracted and analysed by gradient hplc as described above for urine. Metabolites were isolated from similarly prepared extracts of pooled human (20 g), dog (50 g), rabbit (45 g), rat (2 g) and mouse (2 g) faeces homogenates. Owing to similar profiles in male and female dogs, rats and mice, samples were pooled for the two genders. Initially isolated metabolites were further purified by isocratic hplc prior to mass spectral analysis.

Metabolite identification

Metabolite characterization was performed initially by the comparison of retention times on isocratic and gradient hplc systems with authentic standards. To confirm metabolite identities, or to identify components for which no reference standard was available, mass spectral analysis was performed. The radioactive components were analysed by direct infusion (5 μl/min, nebulizer gas pressure 40 psi) into an API III plus mass spectrometer (Perkin Elmer Sciex, Toronto, Canada) fitted with an IonSpray interface utilized in positive ion mode.

In vitro *metabolism*

In vitro metabolism experiments were carried out in the hepatic microsomal fractions from male rat, female rat, rabbit, dog and man. Transplant-quality human liver tissue was obtained from the International Institute for the Advancement of Medicine (Exton, PA, USA). Microsomes were prepared according to the method of Remmer *et al*. (1966) and stored at −80 °C. The concentrations of protein and cytochrome P450 were determined by standard methods (Lowry *et al*. 1951, Omura and Sato 1964).

Microsomal incubations (12 ml) were prepared containing 0.4 μM cytochrome P450, 50 mM TRIS HCl (pH 7.4), 5 mM $MgCl_2$, 5 μM $MnCl_2$, 5 mM isocitric acid, 1 unit/ml isocitrate dehydrogenase and

1 mM NADPH. Sildenafil was added after a 5-min pre-incubation at 37° C to give an initial substrate concentration of 1 μM (selected to avoid saturation of cytochrome P450-catalysed reactions). The mixture was incubated at 37° C and samples (1.0 ml) removed for the analysis of sildenafil for up to 60 min. Single incubations were conducted for each species and metabolism in samples was terminated by extraction into 3 ml ethyl acetate containing the internal standard (UK-89,539). The ethyl acetate extracts were evaporated to dryness and analysed on the hplc system described above for the analysis in animal plasma. Disappearance rate constants (k) in microsomal preparations were determined by linear regression of the log ratio (drug/internal standard) versus time. The *in vitro* microsomal half-life was determined according to the equation $t_{1/2} = \ln 2/k$.

Results

Intravenous pharmacokinetics of sildenafil

Following single i.v. doses of sildenafil to mouse and male rat, the compound exhibited high plasma clearances (50% or greater of liver blood flow) of 91 and 48 ml/min/kg respectively. A marked gender difference in plasma clearance was seen in rat with 13 ml/min/kg in female animals. Moderate plasma clearance values (25% or less of liver blood flow) of 12 and 6.0 ml/min/kg, were obtained in dog and man respectively. The volume of distribution was similar in mouse, male and female rat and man with 1.0, 1.1, 2.0 and 1.2 l/kg respectively. The volume of distribution was greater in dog at 5.2 l/kg. This large volume of distribution in dog resulted in the longest elimination half-life value of 5.2 h (half-life = (0.693 × volume of distribution)/clearance). The high clearance in mouse and male rat resulted in short elimination half-lives of < 1 h. Moderate clearance combined with the lower volume of distribution resulted in half-lives of 1.9 h in the female rat and 2.4 h in man. Pharmacokinetic values after i.v. administration are shown in table 1.

In dogs, urinary excretion of sildenafil accounted for 2.8% of the administered dose, indicating non-renal processes to be the major process of clearance. The piperazine *N*-desmethyl metabolite, UK-103,320 was observed as a circulating metabolite in all species after i.v. administration of sildenafil. In male and female rats, the elimination half-lives for the metabolite (1.1 and 4.3 h respectively) were longer than those of parent compound. After i.v. doses of sildenafil to mouse and dog, plasma concentrations of UK-103,320 were too low to allow accurate calculation of the elimination half-life. In man, the elimination half-life of UK-103,320 was similar to that of parent compound at 2.3 h, whereas the second metabolite, UK-150,564, exhibited a longer elimination half-life of 5.4 h.

Oral pharmacokinetics

Plasma concentrations of sildenafil and UK-103,320 after single p.o. doses to animals and man are shown in figure 2. Data from the higher p.o. doses in rat (45 mg/kg) and dog (20 mg/kg) are shown. These doses were selected on the basis of those used in toxicology studies. In addition, plasma concentrations of the second metabolite, UK-150,564, are shown for man. Summary pharmacokinetic parameters are listed in table 1 and these include p.o. data at the more pharmacologically relevant dose of 1 mg/kg in rat and dog.

T_{max} were ≤ 3 h in all species. Oral bioavailability, calculated by comparison of dose-normalized p.o. and i.v. AUC_∞, was 17% (mouse), 23% (male rat) 44% (female rat), 54% (dog) and 38% (man). The low values in mouse and male rat and to a lesser extent female rat, dog and man reflect metabolic clearance by the liver and a resultant first-pass effect. In dog and man, the half-lives of sildenafil after p.o.

Table 1. Pharmacokinetic parameters for sildenafil after single i.v. and p.o. administration to mouse, male and female rat, dog and man.

Parameter	Mouse ($n = 5$)	Male rat ($n = 3/2$*)	Female rat ($n = 3/2$*)	Dog ($n = 5$)	Man ($n = 3$)
Intravenous dose (mg/kg)	1.0	4.0	4.0	1.0	0.35
Elimination half-life (h)	nd	0.3± 0.1	1.9± 0.1	5.2± 2.1	2.4± 1.0
Plasma clearance (ml/min/kg)	91	48± 11	13± 1	12± 4	6.0± 1.1
Volume of distribution (l/kg)	1.0	1.1± 0.4	2.0± 0.2	5.2± 1.6	1.2± 0.3
Oral dose (mg/kg)	10.0	1.0	1.0	1.0	0.68
C_{max} + (ng/ml)	30	16	136	117± 66	212± 59
T_{max} (h)	0.5	3.0	0.25	1.1± 0.5	1.2± 0.3
Elimination half-life (h)	1.3	0.4	0.9	6.1± 5.8	3.7± 1.4
Oral bioavailability (%)	17	23	44	54± 13	38
Ratio C_{max} sildenafil/UK-103,320	4.8	1.0	8.5	6.9	2.1

* $n = 3$ for intravenous dose, $n = 2$ for oral dose.

+ Normalized to 1 mg/kg oral dose.

nd, Not determined due to insufficient data.

Values are mean± SD where available. Samples or data have been combined for other parameters, thus preventing statistical analysis.

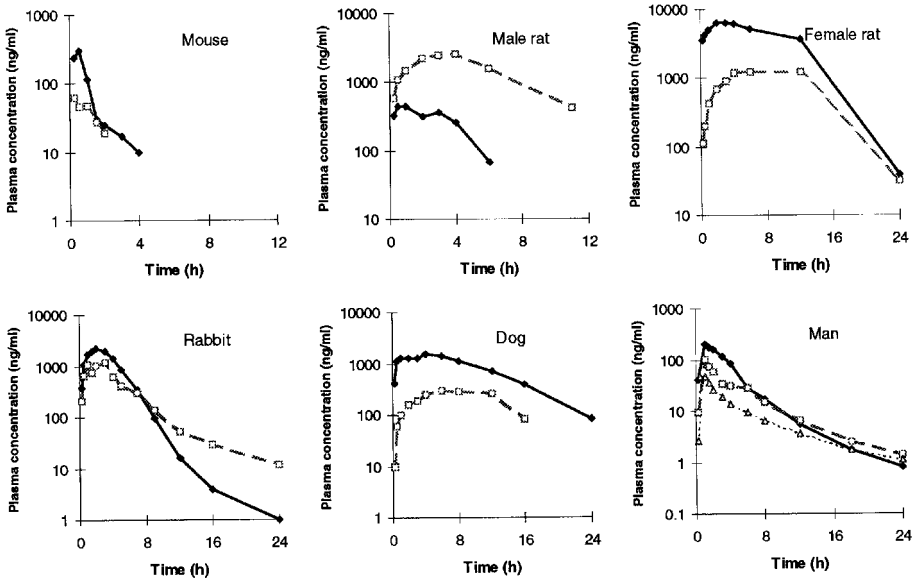

Figure 2. Mean plasma concentrations of sildenafil and metabolites in mouse, male rat, female rat, rabbit, dog and man after single p.o. doses of sildenafil. Administered doses were 10 mg/kg mouse; 45 mg/kg male and female rat; 50 mg/kg rabbit; 20 mg/kg dog and 0.68 mg/kg man. ◆, sildenafil; □, UK-103,320; △, UK-150,564. Data are the means from five mice (pooled samples at each time point), three male and three female rats (individual samples at all time points), seven rabbits (three rabbits providing individual samples at each time point), two dogs and three men (individual samples at all time points).

administration (6.1 and 3.7 h) are slightly longer than the equivalent values after i.v. dosing, suggesting that the rate of absorption limited the overall rate of elimination. In rabbit, at the toxicological dose of 50 mg/kg, the mean C_{max} ($n = 3$) was 2190 ng/ml, observed at 2.0 h and the terminal elimination half-life was 1.8 h.

Comparison of p.o. pharmacokinetic parameters at the different dose levels examined in dog and rat show approximately linear increases in systemic exposure

Table 2. Oral pharmacokinetics of sildenafil in rat and dog at pharmacological and toxicological dose levels.

Species		Oral dose (mg/kg)	C_{max} (ng/ml)	AUC (ng.h/ml)	Ratio C_{max} sildenafil/UK-103,320
Male rat	($n = 2$)	1	16	54	1.0
	($n = 3$)	45	477	2300	0.2
Female rat	($n = 2$)	1	136	252	8.5
	($n = 3$)	45	6620	116000	5.0
Dog	($n = 5$)	1	117	842	6.9
	($n = 2$)	20	1570	18250	5.2

in dog and male rat (table 2). For the 45-fold increase in dose in male rat, increases in AUC and C_{max} are ~ 43- and 30-fold respectively, and the 20-fold dose increase in dog results in 22- and 13-fold increases in AUC and C_{max}. However, clear evidence is observed for the clearance process of sildenafil being capacity limited in the female rat, with a disproportionately high increase in AUC of 460-fold for the 45-fold increase in dose. This saturation has little influence on C_{max}, with a 49-fold increase being observed. In man, the pharmacokinetics of sildenafil are approximately proportional to dose across the clinical dose range (Muirhead *et al*. 1996).

In all species except the male rat, the plasma concentrations of the *N*-desmethyl metabolite, UK-103,320, was less than that of parent compound with C_{max} ratios of sildenafil to UK-103,320 (tables 1 and 2) between 1.9 (rabbit) and 8.5 (female rat). In the male rat at the 45 mg/kg dose level, concentrations of UK-103,320 were significantly greater than concentrations of parent compound with a C_{max} ratio = 0.2. In man, plasma concentrations of the second metabolite, UK-150,564 were lower than those of UK-103,320 with a mean C_{max} = 49 ng/ml. AUC_{∞} for UK-103,320 and UK-150,564 in man ($n = 3$) following p.o. administration were 55 and 27% relative to sildenafil.

Plasma protein binding

The binding of sildenafil to plasma protein was independent of concentration over the concentration range 0.01–10 μg/ml. The mean proportion of drug bound ($n = 8$) was 94 (range 93–94), 95 (94–95), 91 (90–92), 86 (83–87) and 96 (96–97)% for mouse, rat, rabbit, dog and human plasma respectively. The mean proportion ($n = 2$) of UK-103,320 bound to plasma protein at 1 μg/ml was 94, 89, 89, 86 and 95% for mouse, rat, rabbit, dog and man respectively.

In vitro *rates of metabolism*

Disappearance half-lives for sildenafil in hepatic microsomes from male rat, female rat, rabbit, dog and man were 2, 129, 113, 38 and 45 min respectively. No disappearance was detected in the absence of NADPH. In all species, disappearance of parent compound was accompanied by the appearance of a component that had the same hplc retention time as authentic UK-103,320.

Excretion studies

The recoveries of radioactivity in urine and faeces following p.o. administration of [^{14}C]-sildenafil to mouse, rat, female rabbit and dog and i.v. and p.o. administration to male volunteers are shown in table 3; since there was no apparent gender difference, male and female data are combined for mouse and dog. After all

Table 3. Excretion of radioactivity (as % administered dose) in mouse, rat, rabbit, dog and man following single oral or intravenous doses of [^{14}C]-sildenafil.

Species and route of administration	Dose (mg/kg)	Urine excretion (0–120 h)	Faeces excretion (0–120 h)	Total excretion (0–24 h)	Total excretion (0–120 h)
Mouse (3 male, 3 female) p.o.	10	6	85	86	93
Male rat ($n = 3$) p.o.	45	9	88	64	98
Female rat ($n = 3$) p.o.	45	13	82	28	95
Rabbit ($n = 3$) p.o.	50	15	75	62	92
Dog ($n = 2$) p.o.	20	14	73	15	87
Man ($n = 3$) p.o.	0.68	12	79	19	91
Man ($n = 3$) i.v.	0.35	13	76	34	89

administrations to all species, radioactivity was extensively excreted in the faeces over 5 days. Excretion was rapid with a high percentage of radioactivity recovered within the first 24 h in mice, rabbits and male rats. Excretion was slower in female rats, dog and man (28, 15 and 19% respectively in 24 h) in keeping with the longer elimination half-lives and longer gastrointestinal transit times in dog and man.

Faecal metabolites

Recovery of faecal radioactivity by solvent extraction was > 90% for all species and > 96% of applied material was eluted from the hplc column using the gradient conditions described. Metabolite profiles were therefore assumed to be representative of all the biotransformations of sildenafil. Chromatography of faecal extracts revealed a complex mixture of metabolites of [^{14}C]-sildenafil in all species. A representative profile of a pooled human faecal extract (0–72 h) is shown in figure 3. Sildenafil was observed in faecal extracts (retention time ~ 55 min) of mouse (3% of administered dose), rat (3%), rabbit (21%) and dog (16%) but not in man, its identity, when present, being confirmed by API mass spectrometry (parent MH^+ ion, 475, major daughter ions 283 and 311). A major component in faecal extracts from all species, designated M9 (retention ~ 41 min), accounted for 19% of the dose in mouse, 16% in rat, 5% in rabbit, 16% in dog and 22% in man. This component co-chromatographed with authentic standard, UK-150,564, the *N*,*N*-desethyl metabolite of sildenafil, and identity was confirmed by API-mass spectrometry (parent MH^+ ion, 449, major daughter ions 283 and 311). M10 (retention ~ 48 min) showed equivalent retention time to authentic standard, UK-103,320, and identity was again confirmed by API-mass spectrometry (parent MH^+ ion, 461, major daughter ions 283 and 311). This was identified as a faecal radioactive component in all species representing 7, 11, 11, 2 and 3% of orally dosed radioactivity to mouse, rat, rabbit, dog and man respectively. Several radioactive fractions isolated from faecal extracts contained multiple components (e.g. fractions M4, M5, M7 and M8 in human faecal profile), which were subdivided on subsequent hplc separation. One such component in human faeces was designated M8A (retention ~ 37 min) and represented 3% of the administered dose. This component co-chromatographed with authentic standard, UK-331,849 and this identity was confirmed by mass spectroscopy (parent MH^+ ion, 435, major daughter ions 283 and 311). A major component in rat faeces (retention ~ 30 min), representing 20% of the administered dose, co-chromatographed with authentic standard, UK-95,340 and this identity was confirmed by mass spectrometry (parent MH^+ ion, 461, major daughter ions 269 and 297). This metabolite was also observed in dog faeces (8%

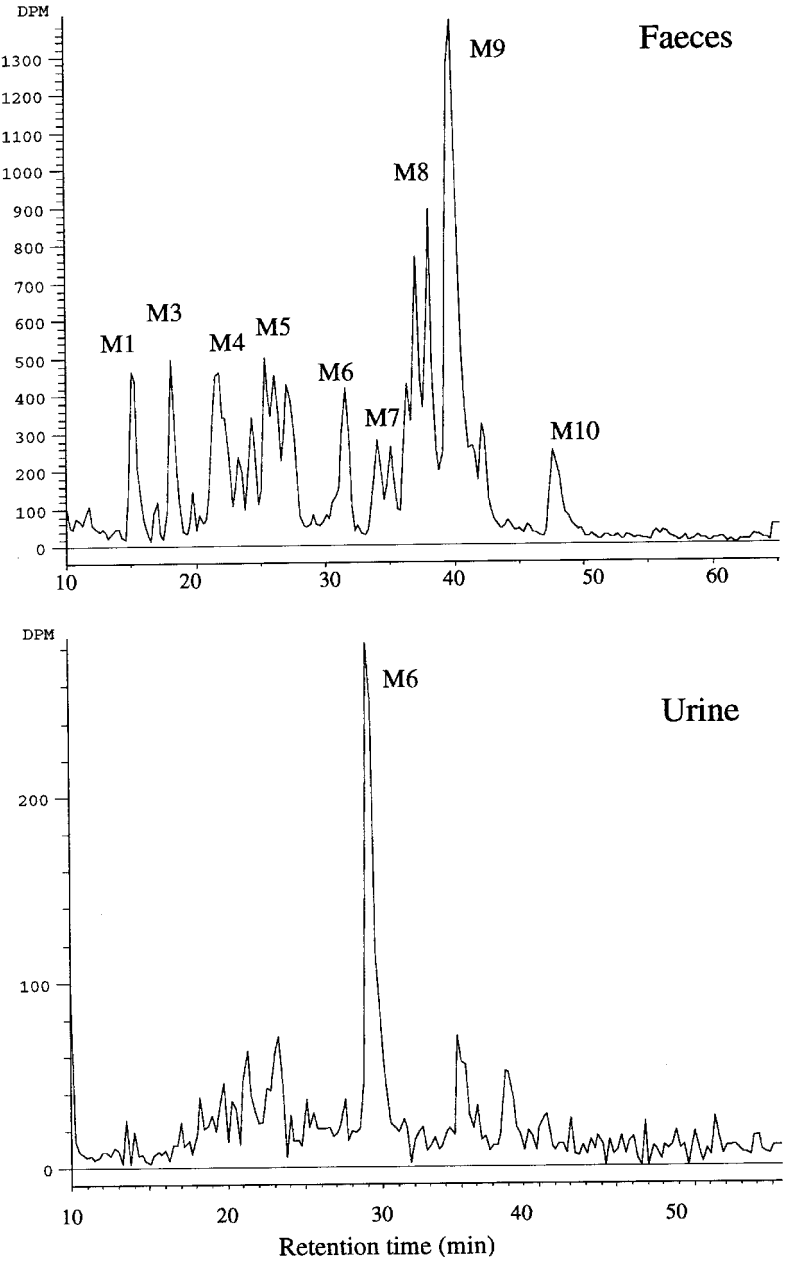

Figure 3. Representative hplc profiles of radioactivity in faecal and urine extracts from man following p.o. administration of [^{14}C]-sildenafil. The gradient hplc system comprised of a 15×0.46 cm Spherisorb 5 μ ODS2 column eluted with 35–70% methanol in 0.05 M aqueous ammonium acetate over 60 min at 1 ml/min.

dose) but was not detected in other species including man. Other individual metabolites accounted for between 2 and 4% of the administered dose in man and represented various oxidations within the molecule and combinations of primary routes. All metabolic pathways observed in man were represented in one or more of the animal species.

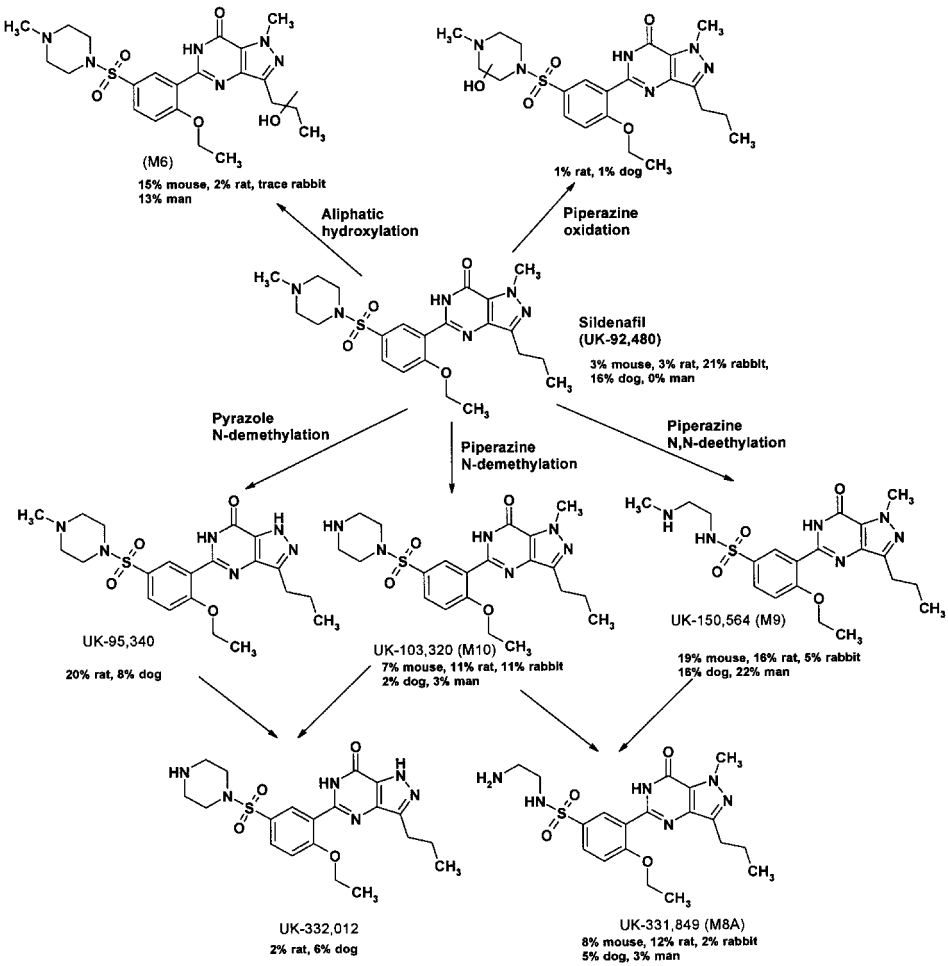

Figure 4. Proposed major metabolic pathways and metabolites of sildenafil. Percentages of dosed radioactivity are given for each metabolite detected in excreta from man and laboratory animals.

Urinary metabolite profile in man

Only one major component was isolated from human urine extracts following p.o. administration of [^{14}C]-sildenafil (figure 3). This component, M6 (retention ~ 32 min), represented 41% of urine (0–24 h) radioactivity and 5% of the administered dose. The metabolite did not co-chromatograph with any of the authentic standards, and mass spectral analysis (data not shown) indicated hydroxylation in the pyrazolo-pyridine part of the molecule, most likely on the 3-propyl substituent. This metabolite was identical (by chromatography and mass spectroscopy) to component M6 observed in human and animal faecal extracts.

The major metabolic pathways of [^{14}C]-sildenafil in animals and man are shown in figure 4.

Circulating metabolites of sildenafil

In man, after p.o. administration, concentrations of radioactivity declined with an identical half-life to that of sildenafil (3.7 h), indicative of formation rate limited clearance of metabolites. In both dog and rabbit, the elimination half-life of

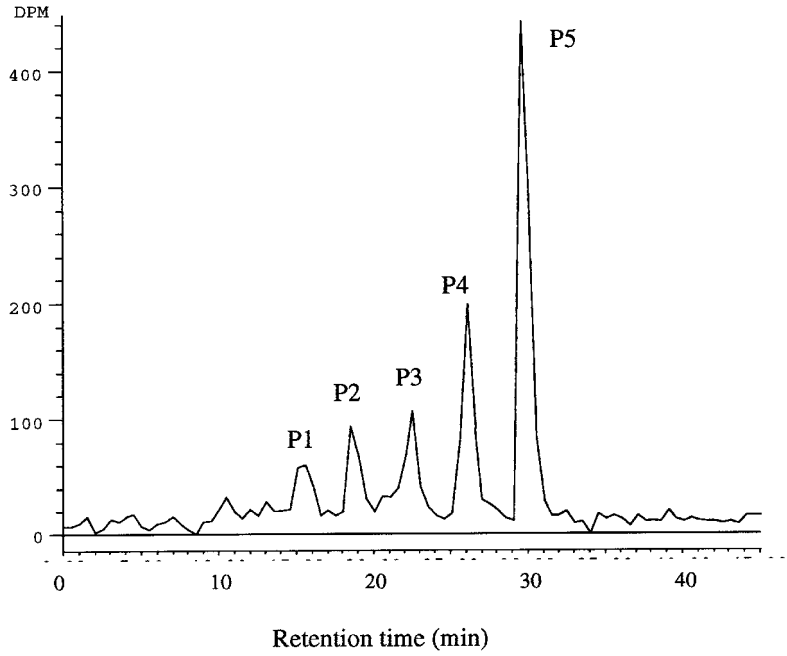

Figure 5. Representative profile of radioactivity in human plasma extract (1 h post-dose) following p.o. administration of [^{14}C]-sildenafil. The gradient hplc system comprised of a 12.5×0.46 cm Spherisorb 5 μ ODS2 column, eluted with 30–80% methanol in 0.05 M ammonium acetate over 30 min at 1 ml/min.

radioactivity was slightly longer (7.3 and 5.1 h respectively), than the half-life of sildenafil determined in the same samples (4.2 and 1.8 h respectively). To complement the specific assay of sildenafil and UK-103,320 in animal and human plasma and UK-150,564 in human plasma, the profile of radioactive components was investigated in human plasma 1 h after the administration of p.o. and i.v. doses of [^{14}C]-sildenafil. The profile of radioactive components in plasma after p.o. administration is shown in figure 5. The major component, designated P5 (47% of plasma radioactivity after p.o. dose and 68% i.v.), was parent compound. The next most slowly eluting component, P4, was identified as UK-103,320 and represented 19 and 9% of plasma radioactivity after p.o. and i.v. doses respectively. Component P3 was identified as UK-150,564 accounting for 13 and 7% respectively of p.o. and i.v. plasma radioactivity. Component P2, represented 9% of p.o. plasma radioactivity (6% i.v.) and was found to have an identical mass spectrum to M6 identified in human and animal excreta. The most rapidly eluting component, P1 (7% p.o., 5% i.v.), was not identified.

Discussion

Absorption of sildenafil from the gastrointestinal tract is judged to be essentially complete by the similar recoveries of radioactivity in the urine of man after i.v. and p.o. administration of sildenafil. In addition, p.o. bioavailability in animal species is consistent with complete absorption and the degree of first-pass metabolism expected from clearance values after i.v. doses. Sildenafil is a weakly basic compound ($pK_a = 6.5$) which is therefore only partially ionized at physiological pH. The

Table 4. Relationship between volume of distribution and plasma protein binding of sildenafil animal species and man.

Species	Volume of distribution (l/kg)	Sildenafil unbound fraction in plasma*	Volume of distribution of unbound drug (l/kg)
Mouse	1.0	0.06	17
Male rat	1.1	0.05	22
Female rat	2.0	0.05	40
Dog	5.2	0.14	37
Man	1.2	0.04	30

* Data are the mean of eight determinations ($n = 2$ at four concentrations).

compound is moderately lipophilic (log $D_{7.4} = 2.7$) resulting in good solubility (Terrett *et al*. 1996). These desirable physicochemical parameters (Smith *et al*. 1990) confer the excellent absorption properties observed with this p.o. agent.

Sildenafil exhibits a volume of distribution in excess of the volume of body water (~ 0.8 l/kg), therefore indicating some tissue affinity, in keeping with its weakly basic nature. Species differences in volume of distribution reflect the differences in plasma protein binding as the volume of distribution of unbound drug (V_{du}) remains relatively constant ($\sim$ 2-fold variation) across the species (table 4). This is in keeping with the high cross-species correlation for V_{du} observed for a large range of drug molecules (Obach *et al*. 1997). Free drug equilibrium between plasma and tissues therefore remains constant (Smith *et al*. 1996). The actual effect of protein binding on total drug distribution to tissues and tissue levels is minimal since at least 90% of the dose resides outside the circulation (i.e. within the tissues) in all cases, with a maximal of 98% in the case of the dog (based on blood volume of ~ 0.1 l/kg). These data on variation in plasma protein binding across the species are consistent with the trend shown for a large number of drugs that demonstrated that man generally has a lower free fraction than animal species (Smith 1987).

Sildenafil is cleared almost exclusively by metabolism, in keeping with its relatively lipophilic nature and hence low renal clearance and excretion due to high tubular reabsorption in the kidney. Indeed, it is likely that the low levels of unchanged drug present in rabbit and dog faeces after radiolabelled doses reflect incomplete absorption (rather than biliary excretion of parent) at the relatively high dose levels used in these studies. The NADPH-dependent metabolism of sildenafil observed in hepatic microsomal fractions from all species suggests that the compound is metabolized by cytochrome P450 enzymes. The relative rates of metabolism are approximately consistent with the clearances observed *in vivo*. The significant gender difference between male and female rat metabolism rate *in vitro* (half-lives of 2 and 129 min respectively) is reflected in the greatly reduced clearance (48 and 13 ml/min/kg respectively) and higher bioavailability observed *in vivo*. Similarly moderate *in vitro* clearance in dog and man is reflected in moderate *in vivo* clearance and bioavailability values in these two species. Saturation of sildenafil clearance is only observed in the female rat over the dose range examined here. Owing to the low first-pass effect in female rats the increase in C_{max} is approximately linear, however there is a 10-fold greater than linear increase in AUC. The saturation is reflected in the plasma concentration-time curve in the female rat (figure 2) which does not exhibit first-order pharmacokinetic behaviour.

Metabolism of sildenafil is complex in all species with up to 16 different metabolites isolated, most of these only representing small fractions of the

administered dose. However, similar metabolic transformations are observed in all species investigated and in general involve the piperazine ring, either via *N*-demethylation, hydroxylation or ring opening. Piperazine *N*-demethylation is well precedented in xenobiotic metabolism, including, for example, the antipsychotic agents clozapine (Jann *et al*. 1993) and olanzapine (Ring *et al*. 1996). However, in contrast with these agents, a piperazine *N*-oxide metabolite was not observed for sildenafil, although an authentic reference standard was available for comparison. In addition, the opening of the piperazine ring of sildenafil is a less commonly observed route of metabolism. Precedents for this route of metabolism were recently reviewed with respect to the elastase inhibitor, L-694,458 (Luffer-Atlas *et al*. 1997) where piperazine scission was also observed. As with sildenafil, this route was observed for L-694,458 and again with and without retention of the piperazine *N*-methyl group. Mono- and dihydroxylated metabolites involving the piperazine ring were observed in small quantities in animal and human excreta and it is possible that they may be intermediates in the formation of UK-150,564. Two successive hydroxylations at adjacent carbons of the piperazine ring have been proposed as a potential mechanism for this ring opening (Baillie *et al*. 1979). *N*-demethylation of the pyrazole of sildenafil, resulting in the metabolite, UK-95,340 is only observed to a significant extent in rat and dog. However, small quantities of metabolites that include this *N*-demethylation in combination with other transformations are observed in man (2% of dose) and mouse and rabbit. No conjugated metabolites of sildenafil were observed in any of the species investigated. The absence of conjugates was confirmed in additional studies with bile duct cannulated male rats (data not shown) in which biliary metabolites were analysed.

In man, sildenafil is the principal component circulating in plasma, and taken together with pharmacological potency it is considered responsible for almost all pharmacological activity observed. Specific analysis for the *N*-desmethyl metabolite, UK-103,320, shows that in all species except the male rat, sildenafil is the major pharmacologically active component present. The very rapid rate of metabolism in male rat results in considerably greater systemic exposure to UK-103,320 than sildenafil ($5\times C_{max}$ and $9\times AUC_{\infty}$) after a p.o. dose. Such a gender difference in cytochrome P450-mediated metabolism in rats is well precedented and is generally unique to this species (Smith 1991).

In conclusion, the pharmacokinetics of sildenafil are consistent with its physicochemical properties. The compound is sufficiently lipophilic and soluble to be well absorbed from the gastrointestinal tract over the clinical dose range (Muirhead *et al*. 1996). Its moderate lipophilic and weakly basic nature result in extensive tissue distribution and clearance by metabolism. Five major routes of metabolism have been identified and all metabolites observed in man are represented in the animal species studied herein.

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